PROTEIN DEGRADATIVE PROCESSES ASSOCIATED WITH ANABOLIC DYSREGULATION IN DIABETIC SKELETAL MUSCLE

A Thesis

by

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ABSTRACT

Skeletal muscle of obese Zucker rats function at higher rates of anabolism when compared to lean littermates, and are resistant to the anabolic effect of exercise. We recently determined that DEP domain containing mammalian target of rapamycin (mTOR)-interacting protein (DEPTOR), a potent negative binding partner of mTOR, is reduced in skeletal muscle of diabetic rats, suggesting that regulation of this protein may participate in the altered protein metabolism. The purpose of this investigation was to assess key regulators of DEPTOR expression, RING-box protein 1 (Rbx1) and beta-transducin repeat-containing protein (β-TrCP), in gastrocnemius muscle of rats with or without type 2 diabetes, with or without *in vivo* resistance exercise. We hypothesized that the reduced expression of DEPTOR would be accompanied by altered expression of Rbx1 and/or β-TrCP. Tissues from thirty male Zucker rats (16 lean [L], 14 obese [F]) collected from previous studies were used for this investigation. Rats were subsequently assigned to sedentary ([S]; 8 LS, 6 FS) or resistance exercise ([E]; 8 LE, 8 FE) groups. Exercised rats participated in four progressive bouts, with increasing repetitions and loads, over 8 days.

Results indicate that DEPTOR mRNA levels were consistent with previously reported protein levels, with reduced content in LE compared to LS ($p<0.05$) and obese groups (FS and FE) not different than LE ($p>0.05$). β-TrCP protein levels were not affected by exercise, but were higher (39%) in obese compared to lean animals; whereas, β-TrCP mRNA content was 113% greater in FE compared to FS ($p<0.05$). There was a

main effect of resistance exercise on Rbx1 protein content, where exercised animals had 30% lower levels of protein than sedentary animals. Further analysis revealed that Rbx1 protein content was suppressed by exercise in lean animals only (49% lower). A main effect of exercise was also observed for Rbx1 mRNA, where exercised animals displayed 47% higher levels than sedentary animals ($p<0.05$).

In conclusion, we speculate that the heightened anabolic function in obese animals may be partly due to increased protein levels of β-TrCP, which may lead to suppressed DEPTOR levels and unrestricted mTOR activity. Additionally, the β-TrCP /DEPTOR interaction may be a key culprit for the reported resistance to anabolic stimuli in diabetic skeletal muscle.

DEDICATION

To my fiancé, Ray Boudreaux, whom I adore. Not only are you my best friend and my number one fan, but you inspire me to work hard and to be the best person I can be each and every day. Your endless love and support (even when writing makes me grumpy) mean the world to me, and for you, I am forever grateful.

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NOMENCLATURE

BW	Body weight
β-TrCP	Beta-transducin repeat-containing protein
DEPTOR	DEP domain containing mTOR-interacting protein
DNA	Deoxyribonucleic acid
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
PCR	Polymerase chain reaction
Rbx1	RING-box protein 1
RNA	Ribonucleic acid
SCF	Skp1-Cullin1-F-box protein

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INTRODUCTION AND LITERATURE REVIEW

Diabetes - a global epidemic. Type 2 diabetes is one of the most common chronic diseases around the world, and its prevalence continues to escalate due to a general shift in lifestyle toward the consumption of higher calorie diets, reduced physical activity, and increased obesity (66). In addition to insulin resistance and hyperglycemia, type 2 diabetes is a metabolic disease also characterized by hyperinsulinemia, dyslipidemia, and altered protein metabolism, a cluster of abnormalities often referred to as the metabolic syndrome. As the risk of diabetes tends to increase with increasing body mass index (BMI), it is of great concern that, in the US alone, nearly 70% of adults are overweight or obese (BMI≥25.0) (21). As such, the Centers for Disease Control (CDC) reported that 25.8 million (or 8.3% of) Americans were afflicted with diabetes in 2011, and if current trends continue, it is projected that 30% of US adults could have diabetes by the year 2050 (1). At present in in the US, diabetes is the 7th leading cause of death, the leading cause of kidney failure, non-traumatic lower limb amputations, and new cases of blindness, and is a major cause of heart disease and stroke, the number one and four leading causes of death, respectively, among American adults (1). In fact, the risk of death among people with diabetes is roughly twice that of individuals with similar characteristics, but without diabetes. Thus, development of treatments or preventative therapies is of paramount importance for maintaining not only the health of individuals with diabetes, but also for reducing the ever-increasing burden on the health care industry.

Etiology of type 2 diabetes. Due to the seemingly unlimited number of factors that underlie the diseased state, understanding factors leading to insulin resistance and type 2 diabetes has proven problematic. Historically, the focus in diabetes research has been on hyperglycemia, the main clinical manifestation, with disease progression believed to begin with a loss of insulin sensitivity in peripheral (adipose and skeletal muscle) and liver tissues, followed by increased insulin secretion by pancreatic β-cells to maintain glucose homeostasis until, eventually, the β-cells can no longer generate enough insulin to meet tissue demands to overcome insulin resistance (13). In addition to the waning ability of skeletal muscle and adipose tissue to take up glucose from the bloodstream, type 2 diabetes is also characterized by increased hepatic glucose production (HGP) in the post-absorptive state (12- to 16-hour overnight fast) along with a dampened ability of insulin to subdue HGP (insulin resistance) (20). The normal, healthy response to increased gluconeogenesis is compensation by decreasing glycogenolysis, due to hyperinsulinemia, thereby maintaining hepatic glucose output (referred to as hepatic autoregulation) (39). However, with type 2 diabetes it is thought that a breakdown of this hepatic autoregulation is a main contributor to the increased HGP and output. Furthermore, the elevated HGP observed in type 2 diabetes has been attributed to elevated circulating plasma free fatty acids (FFA) (46), hyperglucagonemia (3), increased levels of precursors to gluconeogenesis, such as lactate, alanine, and glycerol (9, 10), heightened sensitivity to glucagon (49), and reduced sensitivity to insulin (9).

It is well established that skeletal muscle is the primary site of insulin-stimulated glucose uptake, accounting for about 90% of whole-body glucose metabolism (67), thus making muscle an important player in the pathogenesis of insulin resistance. Glucose enters skeletal muscle cells by facilitated diffusion through glucose transporter protein isoform 1 (GLUT1) and GLUT4. While GLUT1 resides in the plasma membrane and is thought to mediate basal glucose transport, GLUT4 increases glucose uptake in muscle as the protein translocates from its intracellular storage compartment to the membrane in response to insulin or muscle contraction stimuli (32).

As GLUT4 has an essential role in insulin-mediated glucose transport, and type 2 diabetes is often recognized by elevated plasma glucose levels, it is not surprising that numerous research studies have been carried out to examine the effects of altered GLUT4 expression with diabetes. In transgenic mice exhibiting overexpression of human GLUT4 there has been an observed augmentation of skeletal muscle glucose transport, short-term, in the presence of insulin and muscle contraction (6, 32). Conversely, genetic disruption of GLUT4 in skeletal muscle has reportedly induced insulin resistance and glucose intolerance in mice (81). Although defects in GLUT4 expression have not been proven to cause the insulin resistance associated with type 2 diabetes, genetic overexpression or contraction-induced elevation of GLUT4 expression do appear to improve insulin sensitivity in rodents and humans with diabetes (6, 32, 81).

Type 2 diabetes is not due to 'broken' glucose regulation. Recent insight has provided exciting new possibilities that type 2 diabetes is not due to a breakdown of glucose regulation, but perhaps to some other underlying feature of metabolism which

leads to a compensatory shift in glucose metabolism. Mitochondria are critical organelles for energy production for nearly all cellular processes, and their ability to switch between oxidation of glucose and fat requires their proper functioning. David Kelley and colleagues (43, 61, 62) have demonstrated that one such factor in glucose dysregulation is due to altered mitochondrial function. They reported that subjects with type 2 diabetes and obesity have smaller and less efficient (i.e., diminished bioenergetic capacity) skeletal muscle mitochondria compared to lean subjects (43). Since then, a plethora of studies has emerged indicating that altered glucose regulation features a complex interaction between cellular processes in metabolism leading to a directed reduction of glucose uptake in the cell. Those types of studies have led to an altered perception regarding the etiology of the disease, and while the cure remains distant, addressing other aspects of the cell leading to this glucoregulatory disorder appears promising.

It has been purported that insulin resistance in diabetic skeletal muscle is due to an elevated rate of lipid oxidation that is unable to match an excessive FFA uptake, leading to accumulation of intramyocellular lipid metabolites associated with triacylglycerol, including diacylglycerol (DAG), long-chain fatty acyl-CoA, and/or ceramide (11, 37, 65, 70). Those compounds may affect muscle and whole-body glucose (and protein) metabolism by interfering with insulin signaling through activation of serine/threonine kinases. Itani et al. (2000) conducted a study to investigate protein kinase C (PKC) involvement with excessive serine/threonine phosphorylation of the insulin receptor in skeletal muscle of insulin resistant patients (38). Results of that study

indicated that insulin receptor tyrosine kinase activity was reduced as a result of hyperphosphorylation on serine/threonine residues, along with an increased PKC activity in insulin-resistant skeletal muscle, leading to the postulation that the decline in tyrosine kinase activity of the insulin receptor may be caused by serine/threonine phosphorylation by PKC (38).

Altered protein metabolism in type 2 diabetes. Insulin-mediated glucose uptake and protein anabolism are implicated in the same signaling pathway, and considering type 2 diabetes is accompanied by faulty glucose metabolism, it is fitting that alterations in anabolic processes are also observed with the diseased state. To expand on that notion, Fluckey et al. (22) demonstrated that insulin-mediated alterations of PKC activity in diabetic muscle may not only disrupt glucose metabolism, but may also play a role in augmented anabolic responses to insulin, *in situ*. Of particular interest is that inhibition of PKC using a pharmacological inhibitor not only reduced PKC activation following insulin stimulation, but also normalized protein metabolism (22). Further, using a pharmacological agonist that is known for disruption of glucose metabolism (12), insulin responses to anabolism in normal muscle were augmented (22), suggesting that alterations of upstream signals in the signal transduction pathway differentially affect downstream metabolic pathways. Those studies demonstrated a direct link between glucoregulatory and anabolic pathways that respond in an inverse manner (i.e., the insulin signal leads to upregulation of one pathway while downregulating another).

Treating type 2 diabetes and insulin resistance. Strategies for the treatment of type 2 diabetes have almost exclusively been (and largely, still are) focused on reversing

hyperglycemia by stimulating glucose uptake, even in the face of insulin resistance. Such therapies have had limited success, but have proven generally only successful for managing diabetic symptoms, rather than curing the disease itself. There have been many strategies related to the treatment of individuals with type 2 diabetes and insulin resistance, from relatively non-invasive pharmacological approaches (14, 45) to highly invasive bariatric surgeries (2, 59). Those methods seem to have limited, but effective outcomes on the treatment of the disease. Other approaches have been to manage nutrient intake, as reductions of dietary sugar appear to assist patients with diabetes in maintaining manageable glycemia (5, 55). Another relatively non-invasive approach to the disease is physical exercise, which appears to have a profound, albeit temporary, impact on insulin sensitivity (reviewed by Goldbidi et al. [30]). In fact, both endurance and resistance exercise training have been shown to have transient effects on glucose tolerance for up to 48 hours post exercise.

Exercise as a treatment for type 2 diabetes. Endurance exercise has long been known to augment insulin action in skeletal muscle of healthy individuals, and several studies have demonstrated improved insulin sensitivity as a result of aerobic training in a variety of subjects, ranging from youth to elderly and healthy to those with type 2 diabetes. In fact, a single bout of aerobic exercise has been shown to stimulate glucose uptake in insulin resistant skeletal muscle that persists for at least 24 hours following exercise (33). Additionally, Manders et al. (48) demonstrated that a single bout of endurance exercise can stabilize the plasma glucose response for the following 24 hours,

reducing the appearance of post-meal hyperglycemic spikes that are significant precursors in the progression of diabetic complications.

Resistance exercise has also been effective for enhancing insulin sensitivity (17, 23). However, it should be noted that the hallmark of resistance exercise training is to facilitate adaptations in skeletal muscle leading to growth. Thus, the use of this important anabolic stimulant may assist with the sarcopenia observed in type 2 diabetics (63). Recently, other studies have emerged indicating that alterations of anabolic features of skeletal muscle may also play a role in glucoregulatory function. Holten and colleagues (36) reported increased glucose clearance in trained legs of patients with type 2 diabetes following a single leg strength-training program consisting of three 30 minute sessions per week for 6 weeks. Additionally, resistance training increased GLUT4 protein content and activities of insulin receptor, protein kinase B (Akt) α/β, and total glycogen synthase (36). However, these alterations seemed to be independent of gains in muscle mass, as only the healthy subjects (without type 2 diabetes) exhibited skeletal muscle hypertrophy. There is also evidence suggestive that the phosphorylation (activation) of the mitogen activated protein kinase (MAPK), believed to play a critical role in mediating cellular events following contractile activity, is altered in diabetic skeletal muscle (41). Consistent with those findings, our group has also reported abnormal protein turnover and a diminished response to resistance exercise-induced anabolic processes in rats with diabetes (52, 53). Those studies have disclosed a direct link for altered signaling of protein metabolism and insulin resistance to glucose uptake.

mTOR complex composition and action. The mammalian target of rapamycin (mTOR), an evolutionary conserved serine/threonine kinase, is a member of the phosphoinositide-3 kinase (PI3K)-related protein kinase family and is of fundamental importance to the regulation of numerous cellular processes, including cell growth, apoptosis, and metabolism (64). mTOR exists in two structurally and functionally distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (64). mTORC1 is made up of mTOR, regulatory associated protein of mTOR (raptor), proline-rich Akt substrate 40 kDa (PRAS40), DEP domain containing mTOR-interacting protein (DEPTOR), and mammalian lethal with sec-13 protein 8 (mLST8, also known as GβL) (47). mTORC2 consists of mTOR, DEPTOR, mLST8, rapamycin-insensitive companion of mTOR (rictor), mammalian stress-activated MAPK-interacting protein 1 (mSin1), and protein observed with rictor 1 and 2 (protor1/2) (47). In response to amino acids, stress, oxygen, energy and growth factor stimuli (for example, insulin and insulin-like growth factor [IGF]), mTORC1 promotes cellular growth by phosphorylating ribosomal protein S6 kinase 1 (p70-S6K1 or S6K1), eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), and sterol regulatory element-binding protein (SREBP) to induce protein synthesis, lipogenesis, and energy metabolism, and inhibits autophagy and lysosome formation by phosphorylating autophagosome initiators autophagy-related protein 13 (ATG13) and UNC-51-like kinase-1 (ULK1) (47). mTORC2, thought to be activated by growth factors, regulates cytoskeleton organization and cell metabolism and survival through phosphorylation of PKC and Akt/protein kinase B and serum- and glucocorticoid-regulated kinase (SGK), respectively (47).

Regulatory role of mTOR in metabolism. The normal physiological response to elevated nutrients (amino acids and glucose) and insulin in the bloodstream following meal consumption is activation of mTOR to promote anabolism as well as storage and consumption of energy. Specifically, mTORC1 induces mRNA translation, increases in cell mass, and lipid synthesis in white adipose tissue, while inhibiting autophagy, and mTORC2 promotes glucose uptake in most cells and glycogen synthesis in muscle and liver, while inhibiting gluconeogenesis in the liver (47). Conversely, fasting causes declines in blood amino acid, glucose, and insulin levels which leads to decreased activation of mTORC1. This downregulation of mTOR suppresses translation, liver glycogen synthesis, and lipogenesis and adipogenesis in adipose tissue, while stimulating autophagy, gluconeogenesis, lipolysis, and proteolysis to maintain cellular energy levels (47). Additionally, inhibiting the action of mTORC1 results in S6K1 inhibition, preventing uncoupling of insulin receptor substrate 1 (IRS1) from the insulin receptor, perhaps improving insulin sensitivity. Considering mTOR regulatory activity is fundamental to so many cellular processes, it is not surprising that deregulation of the mTOR pathway has been implicated in many human diseases, including cancer and diabetes (4).

Obesity and sarcopenia with type 2 diabetes. Obesity often occurs as a result of excessive nutrient intake combined with low levels of physical activity, and the presence of any or all of the above is considered a major risk factor for the development of type 2 diabetes. A surplus of nutrients can disrupt energetic homeostasis by inducing chronic activation of mTORC1, which can contribute to obesity by facilitating excessive

deposits of ectopic fat and in turn promote insulin resistance (82). In skeletal muscle of obese and high-fat-fed rodents, elevated mTORC1 activation was shown to stimulate S6K1-mediated negative feedback on IRS1, impairing insulin signaling and instigating systemic insulin resistance (44, 71). As a result, Akt activation was blunted, glucose clearance and glycogen synthesis were diminished, and liver gluconeogenesis and release of glucose into the bloodstream were increased, thereby exacerbating the existing hyperglycemia and hyperinsulinemia.

Individuals with type 2 diabetes have smaller muscles than one would predict based on overall mass. This condition, known as sarcopenic obesity (63), is problematic because smaller muscles not only lead to reduced storage capacity for glucose disposal, but also to decrements in skeletal muscle function and strength. Our group recently reported diminished skeletal muscle mass in obese Zucker rats, even following resistance exercise, that was not due to declines in muscle protein synthesis (53). As a follow-up, we analyzed protein members of the mTOR signaling pathway as possible candidates for the reduced anabolic response (52). Differences were not observed between obese and lean animals for upstream mTOR signals; however, protein levels of DEPTOR, an endogenous inhibitor of mTOR and associated with numerous cancers (58, 74), were significantly reduced in obese versus lean rats, making ours the first group to implicate DEPTOR expression as a potential regulator of the anabolic response to exercise in skeletal muscle (52). Since the observed upregulation of mTOR may be due to the degradation of DEPTOR, understanding the mechanisms for this protein's breakdown is of critical importance.

DEPTOR regulation and the ubiquitin proteasome system. The ubiquitin proteasome system (UPS) is critical to numerous cellular processes, from cellular growth to apoptosis, as it regulates a breadth of regulatory proteins via ubiquitin-mediated degradation by the 26S proteasome (35). The UPS is known to involve three key enzymes critical to its proper functioning: an ubiquitin-activating E1 enzyme, an ubiquitin-conjugating E2 enzyme, and an E3 ubiquitin ligase (51). Of these enzymes, the E3 ligase is the one that provides substrate specificity for ubiquitination and subsequent degradation of target proteins (51).

RING ligases are one of two major classes of ubiquitin E3 ligases (HECT domain E3s are the other major class, see [50] for review), and facilitate the direct transfer of ubiquitin from an E2-bound ubiquitin to the targeted substrate. One of the largest and most studied subclasses of RING E3 ligases is the cullin-RING ligases (CRLs), of which the SCF (Skp1-Cullin1-F-box protein) E3 is a member. The SCF complex (Figure 1) is made up of four subunits: three are static, S-phase kinase-associated protein-1 (Skp1), cullin 1 (CUL1), and RING box protein 1 (Rbx1, also known as regulator of cullins 1 [Roc1]), and one is variable, referred to as the F-box protein (26). The CUL1 subunit acts as a molecular scaffold that interacts with Skp1, the adaptor subunit, at the amino terminus and with Rbx1 and an E2 enzyme at the carboxyl terminus (8). Skp1 also binds to an F-box protein, of interest in this study is beta-transducin repeat-containing protein (β-TrCP), which in turn recognizes the target substrate (in this study, DEPTOR) (8). The F-box protein binds the substrate and presents it in close proximity to the E2 enzyme to ensure transfer of ubiquitin to the

targeted protein (26, 73). Multiple rounds of ubiquitination ensue, and then the polyubiquitinated substrate is targeted for proteasomal degradation.

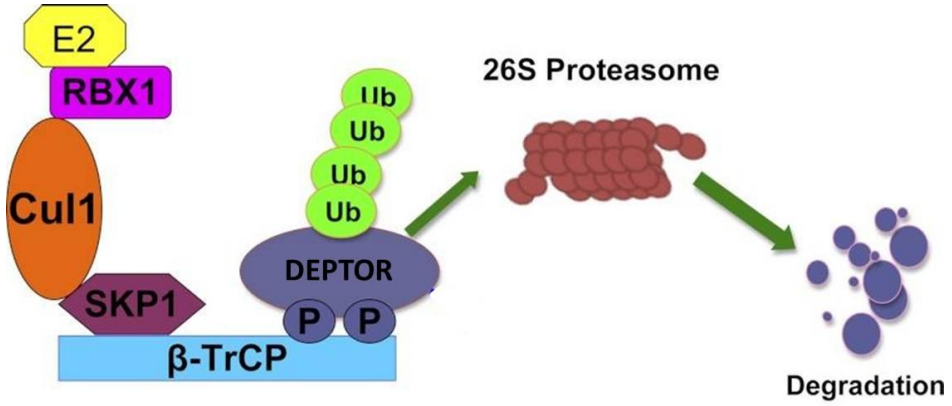

Figure 1. SCF complex targeting DEPTOR for degradation by the 26S proteasome. Ub, ubiquitin; P indicates phosphorylation; E2, Ub-conjugating enzyme. See text for definitions. Adapted with permission from Wang et al. (73).

Rbx1/Roc1 is the catalytic ring component of SCF E3 ligase. Rbx1 is an ubiquitin ligase whose overexpression has been observed in lung, breast, bladder, and colon cancers (40, 72), and has recently been reported to be strongly associated with tumor progression and poor prognosis (72). Of the SCF complex, Rbx1 and CUL1 compose the catalytic core complex which is responsible for recruitment of the E2 enzyme (Figure 1) (60). SCF-type E3 ligase activity is enhanced by post-translational modification of CUL1 by NEDD8 (56), a protein analogous to ubiquitin, and that interaction is precursory to ubiquitination of substrates by SCF. In brief, the RING domain of Rbx1 exhibits conformational flexibility and rotation which has a critical role

for controlling NEDDylation (analogous to ubiquitination, but with specific E1 [NEDD-activating enzyme {NAE}], E2 [UBC12], and E3 [not characterized] enzymes) of CUL1 as a signal to turn on CRL ubiquitin ligase activity (7). Developed as a potential cancer therapeutic agent, MLN4924 is a newly discovered small-molecule inhibitor of NAE (69) that hinders the Rbx1-CUL1 interaction required for NEDDylation (activation) of the SCF E3 complex, and thus prevents the ubiquitin transfer from E2 to target substrate and (what would be) subsequent degradation (79). Of note, Zhao and Sun (79) recently reported an accumulation of DEPTOR following administration of MLN4924 to various cancer cell lines. Those observations support the notion that Rbx1 has an integral role in facilitating $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase activity, and suggest that Rbx1 may serve as a potential negative regulator of DEPTOR.

β-TrCP is the substrate recognition subunit of the SCF E3 ligase complex. β-TrCP is a member of the F-box family of proteins and serves as the substrate recognition subunit of the SCF E3 complex (Figure 1) (73). Recently, Zhao et al. (80) identified DEPTOR as a substrate of the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase. Also implicated in many cancers, though as both oncogene and tumor suppressor (26, 51), β-TrCP has been found to influence mTOR signaling by promoting the ubiquitination and destruction of DEPTOR (74). Gao et al. (28) noted increased expression of DEPTOR and subsequent suppression of mTOR kinase activities in response to β-TrCP depletion. However, these increases in DEPTOR protein levels were not accompanied by increased DEPTOR mRNA levels, suggesting that the accumulation of DEPTOR as a result of β-TrCP depletion occurs through a post-transcriptional mechanism (28).

The mTOR-dependent phosphorylation-driven pathway has recently been shown to be vital for the destruction of DEPTOR via $SCF^{\beta\text{-}TrCP}$ (15, 28, 80). Duan et al. (15), Gao et al. (28), and Zhao et al. (80) conducted experiments that confirmed β-TrCP induction of DEPTOR degradation, and that DEPTOR must be phosphorylated within its degron to recruit β-TrCP. Furthermore, a prerequisite mTOR-induced 'priming' phosphorylation of DEPTOR at a non-degron site, followed by degron phosphorylation by casein kinase 1 (CK1α) was found to be necessary for subsequent β-TrCP recruitment (15, 28), supporting the suggestions that mTOR upregulates its own activity by inducing the degradation of its chief endogenous inhibitor.

Our group has recently demonstrated that DEPTOR regulation is altered in obese male rats exhibiting metabolic syndrome (52). To expand on this work, the present investigation was performed to assess key regulators of DEPTOR expression in skeletal muscle of rats with or without diabetes, with or without an *in vivo* resistance exercise protocol that has been proven (25) to induce anabolism in skeletal muscle. The DEPTOR regulatory proteins chosen for examination in this study were β-TrCP and Rbx1, given each ones integral role in DEPTOR recognition (β-TrCP) and ubiquitin transfer to DEPTOR (Rbx1), thus making them critical to targeting DEPTOR for degradation. The central hypothesis was that changes of DEPTOR expression in skeletal muscle would be accompanied by altered expression of Rbx1 and/or β-TrCP. Specifically, in obese rats, we expected that reduced DEPTOR protein content would be accompanied by increased β-TrCP and/or Rbx1. Also, given that resistance exercise stimulates protein synthesis in lean rats (25, 52, 53), we expected that the decline in

DEPTOR protein levels following exercise would be accompanied by elevated Rbx1 and β-TrCP expression.

MATERIALS AND METHODS

Animals. Tissues used in the present study were obtained from previous studies conducted by our lab group (31, 52, 53). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Texas A&M University. Obese Zucker rats were chosen for use because they are an ideal animal model for understanding the effects of insulin resistance. These animals carry a missense mutation in the leptin receptor gene and manifest symptoms similar to those of patients with type 2 diabetes and metabolic syndrome, including hyperphagia, hyperinsulinemia (see Table 1), hyperleptinemia, hypertriglyceridemia, hypercholesterolemia, glucose intolerance, insulin resistance, angiopathy, and neuropathy (54).

Briefly, thirty 4-month-old lean (*Fa/Fa* or *Fa/fa*) and obese/fatty (*fa/fa*) male Zucker rats (Charles River Laboratories, Wilmington, MA) had free access to food (standard commercial rat chow, Harlan 2016 Teklad Global, Harlan Laboratories, Indianapolis, IN) and water while they were individually housed in a climate-controlled environment with a 12:12-hour light-dark cycle for the duration of the study. Animals were allowed one week for acclimation then were weighed and underwent dual-energy x-ray absorptiometry (DXA; GE Lunar Prodigy DXA [small animal software], Madison, WI) scanning for body composition analysis, specifically total body mass, lean body mass, fat mass, and bone mass (Table 1). To perform the scans, rats were anesthetized using an intraperitoneal (IP) injection of ketamine hydrochloride (Ketaset; 37.5 mg/kg BW) and medetomidine (Domitor; 0.25 mg/kg BW) cocktail, and then were awakened

by administration of antisedan immediately following the scan. Animals were matched according to body composition within each phenotype (fatty or lean) then assigned to groups that either would or would not undergo resistance exercise. Specifically, rats were in one of four groups: fatty sedentary (FS; n=6), lean sedentary (LS; n=8), fatty exercised (FE; n=8), or lean exercised (LE; n=8). A significant main effect, determined by two-way analysis of variance (2x2 ANOVA), was observed for all DXA-derived variables (Table 1). Relative to the lean phenotype, obese rats showed elevated values for bone mass and percent fat mass and lower values for lean mass (all p-values<0.001), as expected. There were no detectable differences between experimental conditions within phenotypes.

	FS, n=6	FE, n=8	LS, n=8	LE, n=8	Phenotype
DXA					
Body mass, g	618 ± 24^{a}	586 ± 20^{a}	384 ± 9.0^{b}	383 ± 6.0^{b}	$p < 0.001$
Bone mass, g	18.0 ± 0.7^{a}	17.0 ± 0.4^{a}	11.0 ± 0.2^{b}	11.0 ± 0.1^{b}	$p < 0.001$
Lean mass, g	222 ± 7.0^{a}	229 ± 13^{a}	313 ± 7.0^{b}	313 ± 5.0^{b}	$p < 0.001$
Fat mass, g	376 ± 18^{a}	340 ± 8.0^{b}	61.0 ± 6.0^{c}	60.0 ± 2.0^{c}	$p < 0.001$
Fat mass, %	63.0 ± 1.0^{a}	60.0 ± 1.0^{a}	16.0 ± 1.0^{b}	16.0 ± 1.0^{b}	$p < 0.001$
Gastrocnemius					
Wet mass, g	1.47 ± 0.07^{a}	1.41 ± 0.04^{a}	1.83 ± 0.01^{b}	1.79 ± 0.05^{b}	$p < 0.001$
Blood					
Insulin, µU/ml	110 ± 24^{a}	126 ± 18^{a}	20 ± 5^{b}	17 ± 5^{b}	$p < 0.001$
Glucose, mg/dl	159 ± 18^{a}	150 ± 19^{a}	121 ± 11^{a}	129 ± 8^{a}	$p = 0.248$

Table 1. Baseline body composition, endpoint muscle mass, and blood insulin and glucose levels. Lean and obese Zucker rats (n=30) were matched based on lean body mass within each phenotype then grouped into cohorts with conditions of either sedentary or resistance exercise. A significant main effect of phenotype was observed for all DXA variables. Wet mass of gastrocnemius muscle and fasted plasma insulin and glucose concentrations were obtained on the final experimental day. Group means that are significantly different according to SNK *post hoc* analyses or *t*-tests do not share the same letter ($p \leq 0.05$). FS, fatty sedentary; FE, fatty resistance exercised; LS, lean sedentary; LE, lean resistance exercised; DXA, dual-energy x-ray absorptiometry. Values are group mean ± standard error of the mean (SEM). Data obtained with permission from Nilsson et al. (53).

Resistance exercise protocol. Animals assigned to exercise groups (FE and LE) participated in a previously described (24) and well documented (19, 29, 34, 53, 76) resistance exercise protocol to elevate rates of skeletal muscle protein synthesis. In brief, following acclimation, rats underwent six operant conditioning (OC) sessions over a two week period in which they were trained in a dark room to press an illuminated lever in a custom Plexiglas® exercise box. An electric foot shock (<3 mA, 60 Hz, 1-5 V) was employed as negative reinforcement to teach the animals to perform what resembles a traditional squat jump in humans by engaging major leg muscles, including the quadriceps and gastrocnemii, to produce full flexion and extension of the hind limbs in response to the light stimulus. In the final two OC sessions, rats wore unweighted Velcro® vests, fitted over the scapulae, while completing the aforementioned movement. Once operant conditioned, rats were able to complete the exercise with minimal or no requirement for shock.

After training, animals began an eight day voluntary resistance exercise program which consisted of four sessions (RE1-RE4), the first three separated by 48 hours each and the final session 72 hours after the third (Figure 2). Five sets were completed during each of the four sessions, with gradually increasing loads and repetitions (reps) throughout each session and from day to day. RE1 consisted of 30 to 230 grams progressively added to vests over a total of 50 reps, RE2 of 80 to 230 grams over 68 reps, RE3 of 80 to 230 grams over 84 reps, and RE4 of 80 to 280 grams over 92 reps. Thus, excluding body mass, the total cumulative weights lifted increased from 5,500 grams during RE1 to 15,460 grams during RE4. Animals were given two seconds rest

between each repetition and two minutes rest between each set. Rats assigned to sedentary conditions maintained normal cage activity for the duration of the experiment, however, they received the average number of shocks administered to exercised animals in an effort to minimize potential for confounding effects of negative reinforcement on skeletal muscle metabolism. The final exercise bout was completed 16 hours preceding euthanasia.

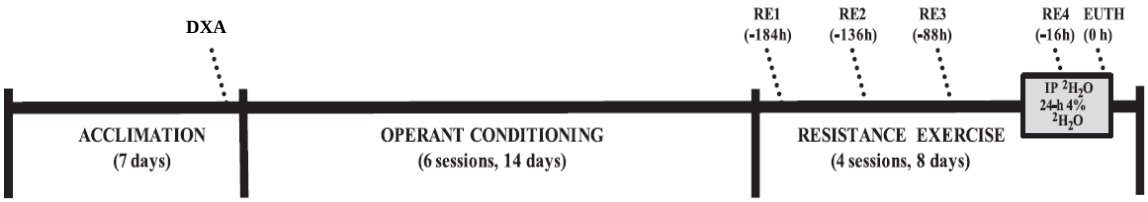

Figure 2. Study design illustration. Following one week of acclimation, animals in exercising groups were operant conditioned to perform a squat-like motion while suited with a Velcro® vest adaptable for gradual increases in weights. With little negative reinforcement, all trained rats completed a resistance exercise (RE) program consisting of four progressive sessions separated by either 48 or 72 hours (RE1-RE3 and RE3-RE4, respectively). The final bout of exercise (RE4) ended 16 hours prior to euthanasia (EUTH). Prior investigators in our lab assessed cumulative muscle protein synthesis (FSR) using deuterium oxide (^2H_2O) methodologies (29, 53), wherein 24 hours prior to EUTH, all animals received an intraperitoneal (IP) bolus injection of 99.9% ^2H_2O and freely accessible 4% ^2H_2O drinking water. DXA, dual-energy x-ray absorptiometry. Figure adapted with permission from Nilsson et al. (53).

Tissue collection. While free access to drinking water remained, food was discontinued four hours prior to tissue harvest. As with previous research (18), all tissues were collected 16 hours following the last exercise session. At that time, rats were anaesthetized with an IP injection of ketamine hydrochloride (Ketaset; 37.5 mg/kg

BW) and medetomidine (Domitor; 0.25 mg/kg BW) then 2 ml of whole blood was exsanguinated via cardiac puncture. The gastrocnemii were subsequently excised, and euthanasia with pentobarbital sodium followed. Fat, blood, and connective tissues were removed before muscles were snap-frozen in liquid nitrogen, pulverized, and then stored at -80°C until used for the present study.

Isolation of protein and western blot analysis. All muscle tissue analyses in the current study were done using mixed gastrocnemius. Western blot analyses were performed as previously described (16) with slight modification. Briefly, pulverized muscle (~60 mg) was homogenized in 360 µl Norris buffer [25 mM Hepes, 5 mM β-glycerophosphate, 200 µM ATP, 25 mM Benzamidine, 2 mM PMSF, 4 mM EDTA, 10 mM $MgCl_2$, 100 mM NF, 10 mM Na_3VO_4, Sigma protease inhibitor cocktail P8340 (Sigma-Aldrich, St. Louis, MO), pH 7.4] and 40 µl of 10% TritonX100, placed on ice for one hour, then centrifuged at 14,000 revolutions per minute for 30 minutes at 4°C to separate the myofibrillar-rich (pellet) and cytosolic protein fractions. The supernatant was decanted into new vials and the protein concentration was determined using bicinchoninic acid (BCA) assay, as described by Smith et al. (68). Based on BCA assay results, samples were stored in 16 µl aliquots at -80°C until fully prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Sample aliquots were thawed on ice, then cytosolic proteins were denatured by the addition of 4 µl of 4X Laemmli buffer (250 mM Tris base, 8% SDS, 40% Glycerol, 0.004% bromophenol blue, 400 mM dithiothreitol [DTT]) followed by 10 minutes of heating at 100°C. 75 µg of cytosolic protein from each sample was loaded onto 10%

polyacrylamide gels (10 cm x 10 cm x 1.6 mm; 37.5:1 acrylamide: bisacrylamide) then separated by electrophoresis in standard electrode buffer (25 mM tris base, 19.2 mM Glycine, 0.1% SDS, pH 8.3) for approximately 1.5 hours at 40 mA (Owl™ P8DS System, Thermo Scientific, Rochester, NY). Experimental groups were equally represented on all gels in an effort to minimize possible confounding effects of gel composition and electrophoresis condition on study outcomes.

In preparation for semi-dry transfer, each gel was removed from SDS-PAGE set-up and placed in Otter transfer buffer (49.6 mM Tris base, 384 mM glycine, 20% methanol [v/v], and 0.01% SDS [w/v]) for 10-15 minutes for equilibration following electrophoresis. Additionally, two stacks of five filter papers (Munktell Filter Paper [Grade 1F], Sweden) were soaked for 10 minutes in Otter buffer, and one polyvinylidene fluoride (PVDF) membrane (Immobilon®-FL Transfer Membrane, 0.45 µm pore size, Millipore Corporation, Bedford, MA) was prepared according to manufacturer instructions. Briefly, PVDF membrane was immersed in 100% methanol for 15 seconds, and then equilibrated for at least 5 minutes in transfer buffer before assembling the transfer stack. All filter papers and transfer membranes were carefully cut so that when stacked, edges were uniform with those of the gel. Assembly of the stack from cathode to anode plates of the transfer box (Owl™ HEP-1 Semi Dry Electroblotting System, Thermo Scientific) was as follows: 5 filter papers, gel, PVDF membrane, 5 filter papers. To ensure an even transfer, air bubbles were removed by carefully (to avoid disturbing the gel and membrane) rolling a glass culture tube over the stack. Proteins were then transferred to the PVDF membrane at 300 mA for 40 minutes. Consistency of loading

and efficiency of transfer were verified with reversible Ponceau S (0.1% Ponceau S [w/v], 5% acetic acid [v/v]) staining of each membrane and Coomassie blue (50% trichloroacetic acid [w/v], 0.1% Coomassie Brilliant Blue [w/v]) staining followed by destaining (50% methanol [v/v], 10% acetic acid [v/v]) of each gel.

Following effective transfer of proteins from gel to membrane, each membrane was incubated for 1 hour in blocking buffer (95% 1X Tris-buffered saline [TBS], 5% dry milk [w/v]), and then incubated overnight at 4°C with gentle agitation in a heat-sealed plastic bag containing 1:1000 primary antibody: blocking buffer. For this study, primary antibodies used were 13 kDa Rbx1 (#4397, Cell Signaling Technology, Inc., Danvers, MA) and 62 kDa β-TrCP (#4394, Cell Signaling Technology, Inc.). A 3 x 5 minute serial wash step using 1X TBS followed, and then each membrane was incubated in 1:2000 anti-rabbit IgG, horseradish peroxidase-linked secondary antibody (Cell Signaling Technology, Inc.): blocking buffer solution for 1 hour at room temperature with gentle agitation. Following a repeated serial 1X TBS wash, each membrane was incubated for 5 minutes in 10 ml of enhanced chemiluminescent substrate (ECL) (equal parts SuperSignal® West Pico Stable Peroxide Solution and SuperSignal® West Pico Luminol Enhancer Solution) (Thermo Scientific, Rockford, IL), then protein bands were developed with a CCD camera mounted in a FluorChem™ SP imaging system (Alpha Innotech, San Leandro, CA). Optical density was determined using AlphaEase FC software (Alpha Innotech), which was set to automatically subtract non-specific binding from densitometry values. Density values were normalized between membranes and expressed as arbitrary units (AU).

Quantitative real-time polymerase chain reaction. Polymerase chain reaction (PCR) analyses were performed at the University of Arkansas in collaboration with Dr. Nicholas P. Greene, PhD, a former doctoral student of Texas A&M University who has been involved with this project from its inception. RNA was extracted with TRIzol® reagent (Life Technologies, Grand Island, NY) as suggested by the manufacturer. Total RNA was isolated, DNase treated, and concentration and purity were determined by fluorometry using the Qubit® 2.0 protocol (Life Technologies). Complementary DNA (cDNA) was reverse transcribed from 1µg of total RNA using Quanta qScript™ cDNA Supermix (Quanta BioSciences, Inc., Gaithersburg, MD). Real-time PCR was performed using the StepOne™ Real-Time PCR system (Applied Biosystems®, Life Technologies) and results were analyzed using StepOne™ Software. cDNA was amplified to 25µg containing appropriate primer pairs and TaqMan® Universal Mastermix (Applied Biosystems®, Life Technologies). Samples were incubated at 50°C for 4 minutes, followed by 40 cycles of denaturation, annealing, and extension at 95°C, 60°C, and 60°C, respectively. TaqMan® fluorescence was measured at the end of the extension step for each cycle. Fluorescence labeled probes for DEPTOR, β-TrCP, Rbx1 (FAM dyes), and 18S (VIC dye) were purchased from Applied Biosystems and were quantified with TaqMan® Universal Mastermix. Cycle Threshold (C_t) was determined and the ΔC_t value was calculated as the difference between C_t value and 18S C_t value. Final quantification of gene expression was calculated using the $\Delta \Delta C_t$ method. Relative quantification was calculated as $2^{-\Delta \Delta C_t}$. Data are presented as fold change of

gene expression relative to 18S ribosomal mRNA. Melt curve analysis was performed at the end of each PCR run to verify that no dimers were formed.

Statistical analyses. The effects of phenotype (fatty vs. lean) and physical activity (sedentary vs. resistance exercised) on expression of Rbx1 and β-TrCP were evaluated using 2x2 ANOVA (SigmaStat 3.5, Systat Software, Inc., San Jose, CA). When significant *F*-ratios were observed, Fisher's LSD *post hoc* analyses were performed to detect differences among group means. Additionally, *a priori* consideration was given for sedentary cohorts between phenotypes (FS vs. LS), as well as for exercise within phenotypes (LE vs. LS, FE vs. FS), thus *t-tests* were performed when appropriate. The level of significance was set at $\alpha=0.05$ for all analyses. All data are presented as group mean ± standard error of the mean (SEM).

RESULTS

DEPTOR protein content is reduced with acute resistance exercise in lean Zucker rats. Our group has previously reported than DEPTOR protein levels were reduced in lean exercised animals relative to their sedentary counterparts (LE vs. LS, p=0.025) (52). Additionally, Nilsson et al. (52) reported no difference in DEPTOR protein levels of obese rats following acute resistance exercise, and that DEPTOR content in obese animals was similar to those of the lean exercised rats (Figure 3).

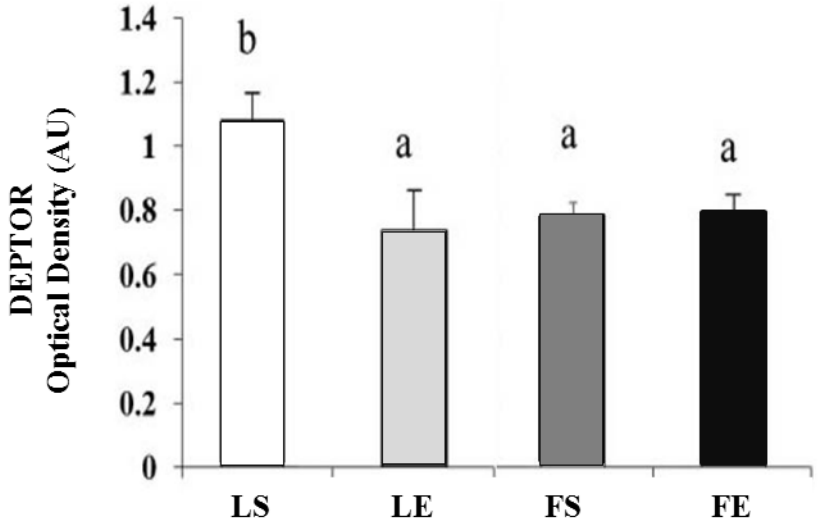

Figure 3. DEPTOR protein content. LS, lean sedentary (n=8); LE, lean exercised (n=8); FS, fatty sedentary (n=6); FE, fatty exercised (n=8). Data are expressed in arbitrary units of densitometry (AU) as group mean ± SEM. Groups with the same letter are not significantly different (p>0.05). Figure adapted from Nilsson et al. (52) and used under permission of Fair Use (USC Title 17 SCC 107).

DEPTOR mRNA is reduced and unresponsive to acute resistance exercise in obese Zucker rats. A main effect of exercise was observed for DEPTOR mRNA in which the exercised animals expressed, on average, 30% less DEPTOR than sedentary animals. There was also a main effect of phenotype in which DEPTOR mRNA was 38% lower in obese compared to lean animals, on average (Figure 4). Further analysis revealed obese sedentary rats (FS) exhibited 44% lower levels of DEPTOR mRNA compared to their lean littermates (LS) ($p<0.001$). With exercise, levels of DEPTOR mRNA for lean rats (LE) were 37% lower ($p<0.001$) than those of their sedentary littermates (LS). By contrast, obese exercised rats were not responsive (FE vs. FS, $p=0.221$) to exercise (Figure 4).

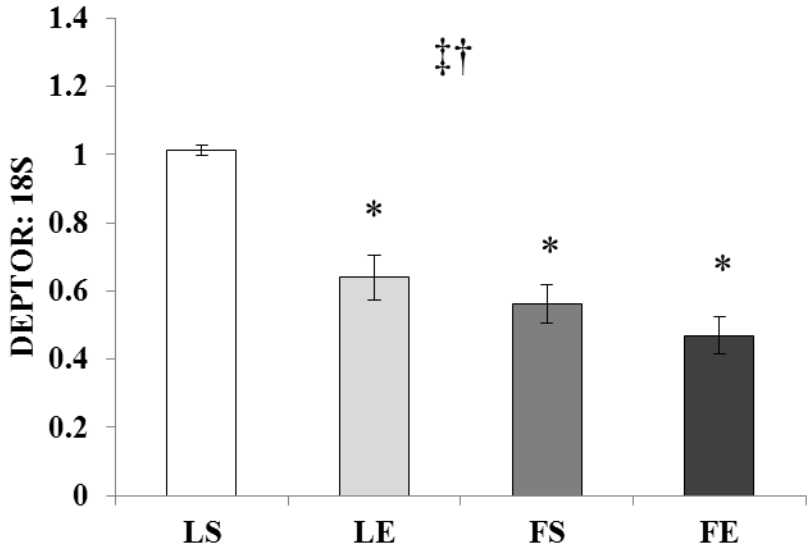

Figure 4. Fold change of DEPTOR gene expression. LS, lean sedentary (n=6); LE, lean exercised (n=6); FS, fatty sedentary (n=6); FE, fatty exercised (n=6). Data are presented as group mean ± SEM. ‡main effect of exercise ($p<0.001$); †main effect of phenotype ($p<0.001$); *different from LS ($p<0.05$).

β-TrCP protein content is elevated in gastrocnemius muscle of obese Zucker rats and is unaffected by acute resistance exercise, regardless of phenotype. There was a main effect of phenotype on β-TrCP protein content in which the level of β-TrCP protein in obese animals was 41% greater (p=0.024) than that of lean animals (Figure 5). However, β-TrCP protein content was not affected by acute resistance exercise.

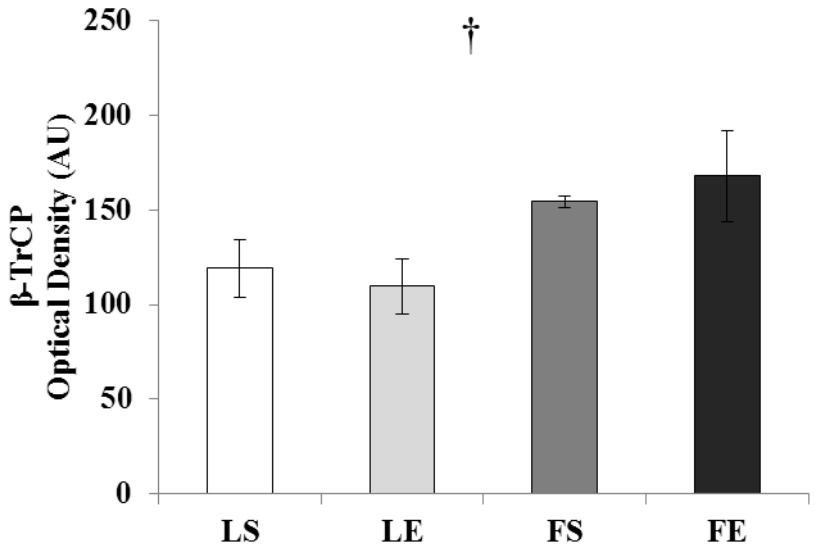

Figure 5. β-TrCP protein content. LS, lean sedentary (n=8); LE, lean exercised (n=7); FS, fatty sedentary (n=4); FE, fatty exercised (n=8). Data are expressed as arbitrary units of densitometry (AU) as group mean ± SEM. †main effect of phenotype ($p<0.05$).

β-TrCP gene expression is elevated with exercise in obese Zucker rats. Results of *a priori t*-tests indicated no statistically significant differences between animals in sedentary groups (p=0.087), but greater expression of β-TrCP mRNA was observed in obese exercised rats compared to their sedentary counterparts (p=0.044). Two-way ANOVA revealed no significant differences (p=0.062 for interaction between exercise and phenotype) (Figure 6).

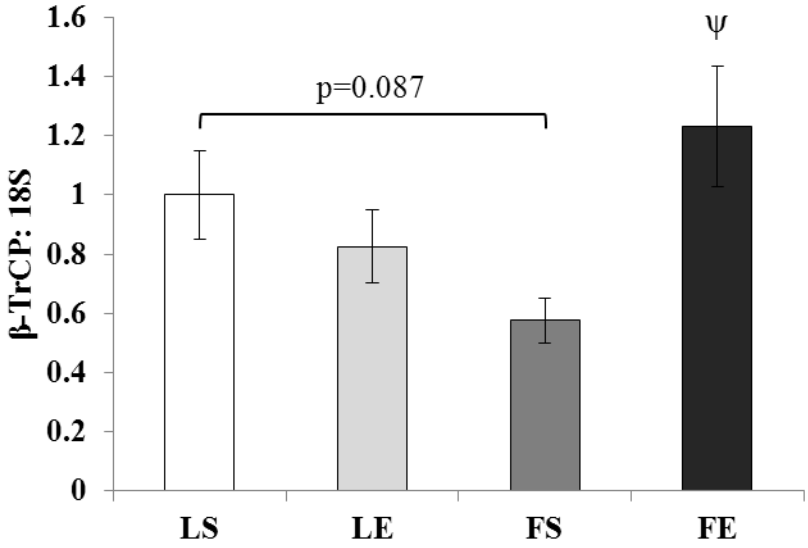

Figure 6. Fold change of β-TrCP gene expression. LS, lean sedentary (n=8); LE, lean exercised (n=6); FS, fatty sedentary (n=4); FE, fatty exercised (n=7). Values are group mean ± SEM. $^{\Psi}$different from FS ($p<0.05$).

Rbx1 protein content is reduced in gastrocnemius muscle of lean, but not obese Zucker rats following acute resistance exercise. A main effect of acute resistance exercise was detected in which animals in exercised groups, on average, exhibited 30% lower Rbx1 protein levels (p=0.036) than those in sedentary groups, regardless of phenotype (Figure 7). Further analysis revealed 49% lower levels of Rbx1 protein (p=0.012) in lean exercised (LE) animals only (Figure 7). There were no differences in Rbx1 protein levels within the obese groups (FS vs. FE, p=0.673).

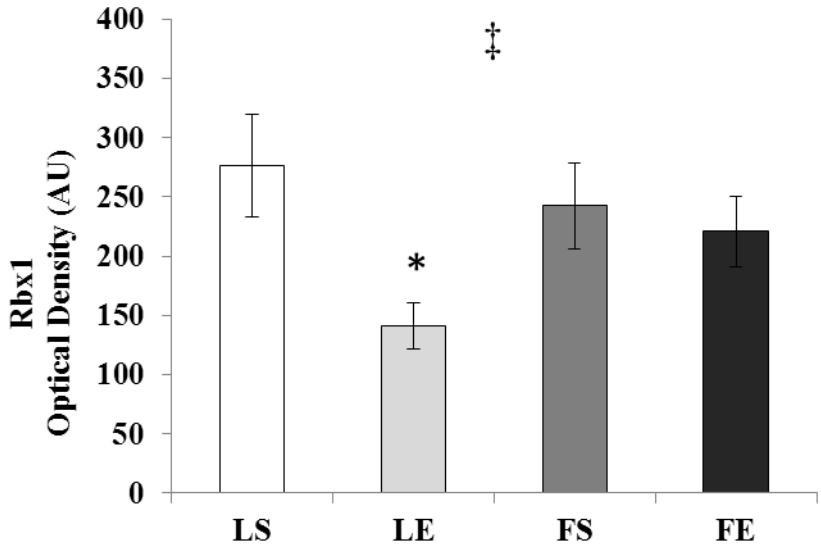

Figure 7. Rbx1 protein content. LS, lean sedentary (n=8); LE, lean exercised (n=6); FS, fatty sedentary (n=6); FE, fatty exercised (n=8). Data are expressed in arbitrary units of densitometry (AU) as group mean ± SEM. ‡main effect of exercise ($p<0.05$); *different from LS ($p<0.05$).

Rbx1 mRNA content is elevated with resistance exercise. There was an observed main effect of exercise, in which animals in resistance exercised groups expressed 47% greater (p=0.032) Rbx1 mRNA content than those in sedentary groups (Figure 8). However, statistical relevance was not achieved within either the lean (p=0.253) or obese (p=0.057) phenotypes.

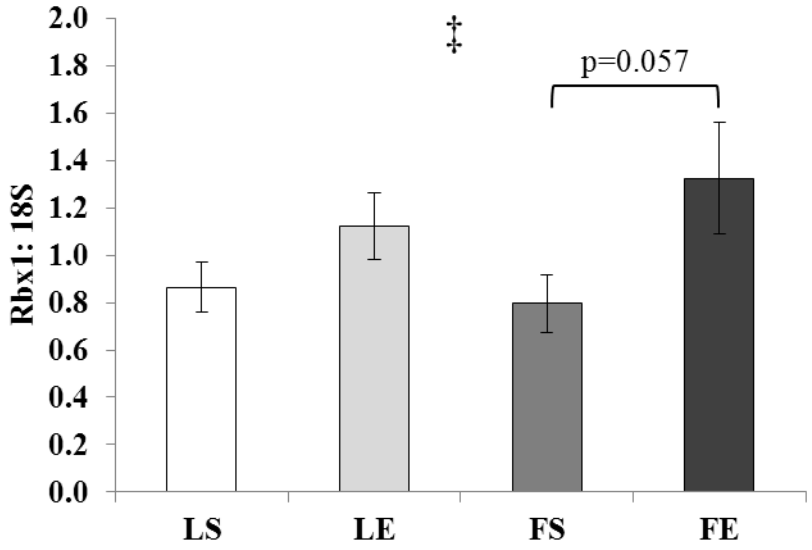

Figure 8. Fold change of Rbx1 gene expression. LS, lean sedentary (n=6); LE, lean exercised (n=8); FS, fatty sedentary (n=4); FE, fatty exercised (n=6). Values are group mean ± SEM. ‡main effect of exercise ($p<0.05$).

DISCUSSION AND CONCLUSIONS

Our group (52) recently reported suppressed DEPTOR expression in obese Zucker rats. Using the same animals and tissues to expand on that work, the primary goal of this investigation was to determine whether the observed DEPTOR levels are coordinated with levels of Rbx1 and β-TrCP, integral members of the SCF-type ubiquitin ligase complex which targets DEPTOR for degradation, in gastrocnemius muscle of rats with or without type 2 diabetes, with or without an acute resistance exercise intervention. Altered levels of the DEPTOR regulatory proteins β-TrCP and Rbx1 have been observed and are associated with poor prognosis in numerous cancers (26, 27, 72, 75); however, to our knowledge these proteins have not been assessed in skeletal muscle, let alone an insulin-resistant model. Thus, our study is the first to demonstrate that expression of β-TrCP protein in skeletal muscle is markedly elevated in the obese Zucker rat as compared to its lean littermate. Furthermore, while it appears that the Rbx1 response to resistance exercise is reduced expression in lean animals, protein content of Rbx1 in the obese animal is rescued following exercise.

Compared to lean animals, obese Zucker rats have markedly reduced levels of DEPTOR protein (52). Given that DEPTOR is an inhibitor of mTOR, and mTOR is integral in promoting protein synthesis, in part through activation of S6K1, it is not surprising that the observed decrements in DEPTOR protein seem to correspond with elevated rates of protein synthesis in the obese Zucker rat (52, 53). Supporting that notion are findings of a 2011 study from Kazi et al. (42) which demonstrated that

DEPTOR knockdown in C2C12 myoblasts, achieved via short hairpin (sh) RNA specific for the DEPTOR mRNA sequence, resulted in increased rates of protein synthesis attributed to mTOR activation of S6K1 and 4E-BP1. Further, these authors conducted DEPTOR knockdown experiments *in vivo* using a mouse hind limb immobilization model, and found that muscle atrophy was prevented with knockdown in the immobilized limb (42). Interestingly, gastrocnemius muscle mass independent of immobilization was unaffected by DEPTOR knockdown alone, suggesting that other contributors in the anabolic process are necessary to promote growth in skeletal muscle. Consistent with Kazi and colleagues (42), our work demonstrated that suppressed DEPTOR levels, elevated rates of muscle protein synthesis, and hyperactivation of mTOR and S6K1 in the obese Zucker rat were not associated with gains in muscle mass (52, 53). Together, it appears that DEPTOR regulation is an important contributor to the preservation of muscle mass under atrophying conditions, albeit unsuccessfully with respect to the obese Zucker rat (52).

The present study expands on previous work (52, 53) by assessing upstream and downstream contributors to DEPTOR expression as possible indicators for the altered content in obese Zucker rats. Results from our work demonstrate that DEPTOR mRNA content (Figure 4) was remarkably consistent with DEPTOR protein content (Figure 3), with obese animals expressing less DEPTOR than their lean counterparts, suggesting that the elevated anabolic response in obese animals may, in part, be due to a diminished message 'availability' for the expression of DEPTOR protein. Thus, a limited capacity for mRNA translation of DEPTOR, coupled with hyperinsulinemia accompanying

metabolic syndrome to augment mTOR activation, may serve to perpetuate the anabolic signal to offset the diminishing muscle mass in these animals.

Furthermore, while mRNA content of DEPTOR was not affected by exercise in the obese animals, it was significantly reduced in lean animals and is consistent with the reduced DEPTOR protein levels following resistance exercise in lean Zucker rats (52). Our group (52) was the first to link DEPTOR as a potential regulator of the anabolic response to exercise in skeletal muscle, and it appears, in part, that alterations of DEPTOR content are via modifications to the availability of DEPTOR mRNA. To that end, Peterson et al. (58) reported a downregulation of DEPTOR protein following activation of mTOR in HeLa cells as well as a downregulation of DEPTOR protein in cancer cell lines with 'constitutive' mTOR signaling. Those authors (58) also demonstrated that reducing mTORC1 activity via shRNA specific for raptor resulted in increased DEPTOR protein and mRNA levels in HeLa cells, which is consistent with what our group (52) has observed using the phosphorylation of S6K1 (at threonine 389) as a surrogate marker of mTORC1 activity. Nonetheless, more work is warranted as to how exercise or obesity alters mRNA content.

The aberrant response to exercise in animals with insulin resistance has been previously documented by our lab group (31, 52, 53). While it appears that altered mRNA content with obesity or exercise has a role in DEPTOR synthesis, the present investigation also explored the possibility that the dysregulation of anabolic processes in obese Zucker rats was due to altered degradation of the DEPTOR protein. Specifically,

we focused on β-TrCP and Rbx1, key regulatory proteins of the $SCF^{\beta\text{-}TrCP}$ complex which is a primary pathway used for the degradation of DEPTOR protein (15, 28, 80).

β-TrCP is overexpressed in many cancers and seems to act as an oncogene, promoting tumor growth and survival (27). Here we demonstrate that obese Zucker rats have elevated levels of β-TrCP protein in skeletal muscle in the basal state when compared to lean littermates (Figure 5). However, it should also be noted that the elevation in protein content does not appear to be a result of altered mRNA content independent of exercise (Figure 6). Additionally, β-TrCP protein content was unaffected by exercise (Figure 5), although β-TrCP mRNA content was greater in the obese following resistance exercise when compared to their sedentary littermates (Figure 6). It is unclear as to why obese but not lean animals would have elevated mRNA content following exercise, but the lack of a response in lean animals is consistent with Zanchi and colleagues (78), who detected no change in mRNA content of β-TrCP in healthy rat plantaris after a 12 week resistance exercise training program. Nonetheless, heightened levels of this important regulatory protein may be contributing to the observed reduction in DEPTOR protein content and the resultant chronic activation of S6K1 by mTOR, thus promoting feedback inhibition of IRS1 and exacerbating insulin resistance. This obesity/metabolic syndrome-specific response warrants further investigation. Frescas and Pagano (26) have suggested that the development of small molecule inhibitors of SCF ligases that target F-box proteins, especially β-TrCP, is essential for the future of cancer therapy. If β-TrCP inhibition allows for the return of normal mTOR signaling

and maintenance by DEPTOR, perhaps controlled anabolic processes and insulin sensitivity could be restored in individuals with type 2 diabetes.

Another important regulator of the $SCF^{\beta\text{-}TrCP}$ complex, Rbx1, is an ubiquitin ligase that is bound to the scaffolding component (CUL1) of this important degradative machinery. Thus, the regulation of this protein may have important implications for the $SCF^{\beta\text{-}TrCP}$-dependent degradation of DEPTOR. Several reports have indicated that independent regulation of this protein has strong implications on the progression of cancers (72, 75, 77). Aside from cancer studies and independent of the $SCF^{\beta\text{-}TrCP}$ complex, Rbx1 has been purported to regulate the ubiquitination and degradation of S6K1 (57), a protein that promotes mRNA translation following phosphorylation by mTORC1 (82). Panasyuk and colleagues (57) demonstrated that overexpression of Rbx1 led to increased S6K1 ubiquitination, while downregulation of Rbx1 promoted stabilization of S6K1 protein in human embryonic kidney (HEK) 293 cells. Although Rbx1 protein levels were not different between lean and obese sedentary rats in the present study, our findings demonstrate a reduction in Rbx1 protein content following acute resistance exercise in lean animals (Figure 7), supporting the notion that reduced Rbx1 promotes S6K1 stability, which likely contributes to the elevated rates of protein synthesis observed in healthy skeletal muscle in response to an anabolic stimulus (52, 53). This reduction of Rbx1 protein levels cannot, however, be explained by a reduction in Rbx1 mRNA levels, as those were no different in lean animals following exercise (Figure 8), suggesting that enhanced degradation of the protein is the cause of the observed decrement. Alternatively, the obese animals seemed to maintain Rbx1 protein

levels in the face of resistance exercise (Figure 7). On average, animals that underwent resistance exercise had higher levels of Rbx1 mRNA (Figure 8), but that main effect of exercise was not sufficient to maintain Rbx1 protein levels in the lean animals (Figure 7). Of particular interest is that Rbx1 protein content was seemingly 'rescued' in the obese exercised animals (Figure 7), and although the mechanism behind this response is unclear, it appears to be a compensatory mechanism attempting to even further reduce the already low DEPTOR protein (Figure 3) and diminished mRNA content (Figure 4) in an unsuccessful effort to augment an anabolic response in muscle after exercise in these sarcopenic animals. More work is necessary to resolve this speculation. Furthermore, it should be noted that the maintenance of Rbx1 in obese animals was not accompanied by reduced total S6K1 protein content (52), which is consistent with the notion that skeletal muscle of obese Zucker rats is 'desensitized' to the anabolic effects of resistance exercise (31, 52, 53).

Recent evidence from our group (52) indicates that the obese Zucker rat experiences a chronic activation of mTOR and S6K1 proteins, suggesting that Rbx1 activity in obese cohorts is centered on the maintained suppression of DEPTOR in these animals. The heightened FSR in the obese Zucker rat, coupled with an inability to augment protein synthesis following exercise (52, 53), may imply that these animals may be "locked" in an anabolic state, suggesting marked differences in proteolytic mechanisms in obese and lean animals. In support of that speculation, we demonstrate here that the obese rats continue to express Rbx1 following resistance exercise, and this is perhaps caused by, and likely contributes to, anabolic dysregulation of skeletal muscle

with diabetes. Future work should focus on the implications of Rbx1 expression as it relates to overall degradative processes in the cell, as well as how conditions of obesity and/or diabetes impact that regulation.

In summary, the present investigation is the first to examine the DEPTOR regulatory proteins β-TrCP and Rbx1 in skeletal muscle of lean and obese Zucker rats. We have previously observed that obese Zucker rats function at elevated rates of anabolism when compared to lean littermates, and thus appear unresponsive to exercises that are known to stimulate anabolism (52, 53). Here, we demonstrate that the heightened anabolic function in obese rats may be, in part, due to increased protein levels of β-TrCP, which may contribute to suppressed DEPTOR levels, resulting in uncontrolled mTOR activity in these animals. Furthermore, the findings herein suggest that the β-TrCP/DEPTOR interaction may be a key culprit for the reported resistance to anabolic stimuli in diabetic skeletal muscle. Results from this study may be of clinical importance. As obesity and diabetes continue to increase in prevalence throughout the world, it is important to consider novel therapeutic targets for these diseased states.

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